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The Detection of Peroxynitrite Using 19F Magnetic Resonance Probes

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Engaged Learning Final Report

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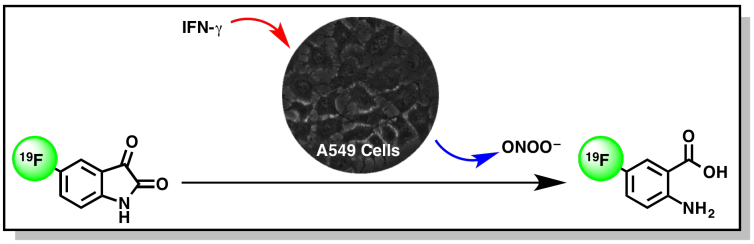
Abstract

The following report presents the work done by Kevin Bruemmer for his Engaged Learning project. The ^{19}F magnetic resonance probes 5-fluoroisatin and 6-fluoroisatin were reacted with $ONOO^-$ to yield anthranilic acid derivatives through a novel oxidative decarbonyaltion reaction. Utilizing ^{19}F NMR spectroscopy techniques, the presence of $ONOO^-$ can be determined through a chemical shift switching signal. 5-fluoroisatin was subsequently used to detect $ONOO^-$ in living lung epithelial cells.



Introduction

Peroxynitrite ($ONOO^-$) is an endogenously present nitrogen oxide species that is formed through the reaction of superoxide and nitric oxide radicals.[1] $ONOO^-$ is highly reactive, and is thought to play a detrimental role in biological systems. Recently, it has been shown that $ONOO^-$ production is increased in patients with cancer,[2] asthma,[3] and cardiac disease.[4]

Specifically, $ONOO^-$ is thought to cause damage in the cardiovascular system, disrupt DNA replication, and be a cause of carcinogenesis (Figure 1). In the cardiovascular system, $ONOO^-$

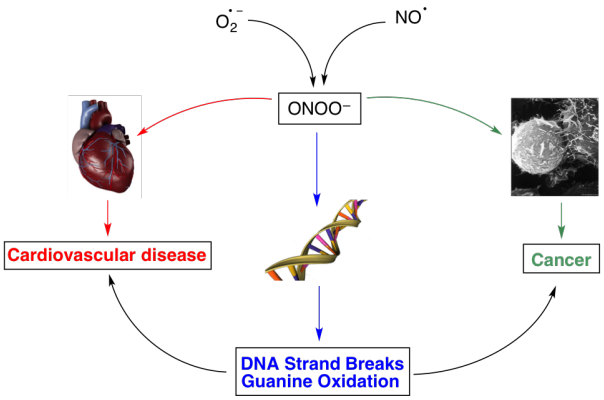


Figure 1: $ONOO^-$ Reactions in Biological Systems

induces cell damage via lipid peroxidation, causes inactivation of enzymes and other proteins by oxidation and nitration, and also activates stress signaling molecules to release apoptosis inducing factors.[1] $ONOO^-$ causes DNA strand breaks that lead to an overworking of DNA repair molecules, ultimately causing a depletion of glycolysis which leads to cardiovascular inflammation. $ONOO^-$ also affects DNA by causing guanine modifications that lead to homologous recombination of DNA, which is recognized as a key initiator of carcinogenesis.[1]

Current methods of $ONOO^-$ detection are limited due to low sensitivity, slow reaction times, and indirect detection methods. The most common method to date for $ONOO^-$ detection is to use antibodies to stain proteins with nitrated tyrosine residues.[5] While this method has proven valuable to understand where $ONOO^-$ is produced in biological systems, it is an indirect method for detecting $ONOO^-$ and is incompatible with living systems. Fluorescent redox-sensitive probes dichlorodihydrofluorescein and dihydrorhodamine have been developed, but have a slow reaction time with $ONOO^-$.[6] Thus, there is a strong need for a direct, specific, and fast reacting detection

method for $ONOO^-$.

^{19}F NMR spectroscopy offers a promising approach for the detection of chemical species in biological systems.[7] ^{19}F NMR has an inherently low background noise for imaging biological specimens, as most of the fluorine is deposited in the skeletal system. The ^{19}F isotope is 100% abundant in nature, making it easy to attach ^{19}F labels on existing chemical scaffolds.

Herein, we report two ^{19}F magnetic resonance probes, 5-fluoroisatin and 6-fluoroisatin, for the specific detection of $ONOO^-$. Fluoroisatins are capable of reacting with $ONOO^-$ through a newly discovered oxidative decarbonylation reaction (Figure 2). Upon reacting with $ONOO^-$, fluoroisatins

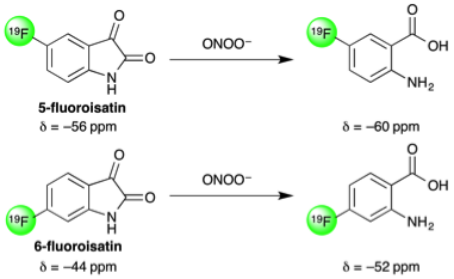


Figure 2: $ONOO^-$ mediated oxidative decarbonylation of 5-fluoroisatin and 6-fluoroisatin

convert to anthranilic acid derivatives. The reaction is capable of being monitored by ^{19}F NMR spectroscopy, enabling the detection of $ONOO^-$.

Experimental

General Methods

5-fluoroisatin was purchased from TCI (Portland, OR) and used without further purification. Deuterated solvents were purchased from Cambridge Isotopes (Cambridge, MA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO). 6-fluoroisatin was synthesized using a previously published procedure.[8] ^1H NMR and ^{19}F NMR spectra were taken on a JEOL ECX series 11.7 T NMR spectrometer in the Department of Chemistry at Southern Methodist University. Chemical shifts are reported in the standard notation of parts per million. High performance liquid chromatography was performed on an Agilent 1100 Series HPLC in the Department of Chemistry at Southern Methodist University.

Peroxynitrite Synthesis

$ONOO^-$ was synthesized from H_2O_2 and isopentyl nitrite in a mixed solvent system, using a procedure slightly modified from a previous description.[9] The solvent system, 4.5 mL of 0.55 M NaOH and 5 mL isopropyl alcohol, was added to an open round bottom flask at room temperature. Then, H_2O_2 (30% wt. in H_2O, 0.24 mL, 2.4 mmol, 1.2 equiv) was added to the solvent system, followed by isopentyl nitrite (0.27 mL, 2.0 mmol, 1 equiv). The reaction was stirred for 15 min at room temperature, and then quenched with 5 mL of 5 M NaOH. Immediately to the quenched reaction mixture, MnO_2 (10 mg, 0.115 mmol) was added to decompose the unreacted H_2O_2. The quenched reaction mixture was stirred for 5 additional min and then filtered. The filtrate was poured into a 250 mL separatory funnel and washed four times with 40 mL CH_2Cl_2. The concentration of $ONOO^-$ in the remaining aqueous layer was measured by UV/Vis spectrometry using an extinction coefficient at 302 nm of 1670 M^{-1} cm^{-1}. This method typically provides $95 - 120$ mM $ONOO^-$ in an alkaline solution.

NMR Spectroscopy Procedure

^{19}F NMR spectra were acquired using a D_2O lock in a reference tube at 25 °C. A 0.1% solution of trifluoroacetic acid in D2O in the insert tube was used as an internal reference for response and selectivity measurements, and a 0.001% solution was used for cell testing. The number of scans was 128 for response and selectivity measurements and 7300 for cell testing. NMR spectra were processed using Delta NMR spectroscopy and plotted using Microsoft Excel. NMR spectrum were processed with DC balance, hamming, Fourier transform, machine phase, baseline correction, and absolute value functions.

Selectivity Test Procedures

Selectivity tests were conducted using ^{19}F NMR spectroscopy. Various reactive sulfur, oxygen, and nitrogen (RSON) species were tested. Each test was performed at 1.5 equivalents of the RSON species to the probe, except for GSH, which was tested at 10 equivalents. After preparation, each reaction mixture was added to an NMR tube, and then 128 ^{19}F NMR scans were acquired and processed according to the general methods for NMR spectroscopy section.

$ONOO^-$: 3.9 µL of 96 mM $ONOO^-$ (final concentration 750 µM) was added to 492 µL of 500 µM isatin in 100 mM HEPES buffered at pH 7.39.

·OH: 1 mg $Fe(ClO_4)_2$ (final concentration 750 µM) was added to an 8 mL solution of 500 µM isatin and H_2O_2 (final concentration 750 µM) in sodium phosphate buffer (100 mM, pH 7.4) at room temperature as described previously.

Glutathione (GSH): GSH testing was conducted at a 10:1 equivalence. 1.5 mg GSH (final concentration 5 mM) was added to a solution of 500 µL of 500 µM isatin in 100 mM HEPES buffered at pH 7.39.

PROLI NONO-ate: A solution of 237.8 mM PROLI NONO-ate was made in 0.01 M NaOH and stored at 0 °C during the day of the experiment. 1.6 µL of this solution was added to a solution of 497 µL (final concentration, 750 µM) of 500 µM isatin in 100 mM HEPES buffered at pH 7.39.

$Na_2N_2O_3$: 1.2 mg Angeli's salt ($Na_2N_2O_3$) (final concentration 100 mM) was added to 100 µL of 0.01 M NaOH. On the same day, 3.75 µL (final concentration 750 µM) of the resulting 100 mM solution of Angeli's salt was added to 497 µL of 500 µM isatin in 100 mM HEPES.

O_2^-: A 750 µM solution of superoxide was made by adding 1.5 mg KO_2 to 28 mL of 500 µM isatin in HEPES buffered at pH 7.39.

tBuOOH: 0.5 µL of 0.728 M tBuOOH (final concentration 750 µM) was added to 499.5 µL of 500 µM isatin in 100 mM HEPES buffered at pH 7.39.

GSNO: 1.3 mg S-nitrosoglutathione (final concentration 750 µM) was added to a solution of 5 mL 500 µM isatin in 100 mM HEPES buffered at pH 7.39.

NO_2^-: 0.5 mg of $NaNO_2$ was added to a solution of 10 mL of 500 µM isatin in 100 mM HEPES buffered at pH 7.39.

OCl^-: 2.5 µL of 0.15 M NaOCl (final concentration 750 µM) was added to a solution of 500 µL of 500 µM isatin in 100 mM HEPES buffered at pH 7.39.

$KHSO_5$: 1.2 mg of Oxone (final concentration 750 µM) was added to a solution of 10 mL of 500 µM isatin in 100 mM HEPES buffered at pH 7.39.

H_2O_2: 3.75 µL of 100 mM H_2O_2 solution (final concentration 750 µM) was added to a solution of 500 µL of 500 µM isatin in 100 mM HEPES buffered at pH 7.39.

HPLC Studies

High performance liquid chromatography (HPLC) studies were conducted to confirm the results of the ^{19}F NMR experiments. Stock solutions of 10 mM 5-fluoroisatin and 5-fluoroanthranilic acid were made in acetonitrile (MeCN). 25 µL of the MeCN stock solution was added to 475 µL 20 mM HEPES (final concentration 500 µM) and then injected into the HPLC, and detected with a UV/Visible detector at 245 nm. The mobile phase consisted of MeCN and H_2O at a flow rate of 1.000 mL/min. A gradient of the mobile phase was implemented over the course of a 20 minute collection, with an isocratic solution of 0% MeCN for 3 minutes, a gradient from 0% MeCN to 40% MeCN for seven minutes, a gradient from 40% MeCN to 100% MeCN for five minutes, and then an isocratic solution of 100% MeCN for five minutes. 5-fluoroanthranilic acid has a retention time of 0.8 minutes and 5-fluoroisatin has a retention time of 8.2 minutes under these conditions. Selectivity studies were performed as described above and HPLC traces were acquired after 15 minutes. The peaks for both 5-fluoroanthranilic acid and 5-fluoroisatin were quantified using the standard calibration curves and corrected using the values in the blank HPLC traces.

Cell Testing

Cell experiments were prepared by Sarah Morris, a graduate student in the Biology department at Southern Methodist University. After preparation, a sample of the cellular media was transferred to an NMR tube and spectra were acquired with 7300 scans at 11.7 T. Quantification of the conversion of 5-fluoroisatin to the corresponding 5-fluoroanthranilic acid was performed on each of the acquired ^{19}F NMR spectra. The concentration of 5-fluoroanthranilic acid was determined by comparison to the trifluoroacetic acid insert standard. To confirm the production of $ONOO^-$ by the cells, imaging was conducted using an EVOS-fl fluorescence microscope with dihydrorhodamine as a fluorescence $ONOO^-$ probe.

Results and Discussion

Response Measurement

A pure sample of each fluoroisatin and the corresponding anthranilic acid was analyzed using ^{19}F NMR spectroscopy. The chemical shifts were determined for 5-fluoroisatin ($\delta = -56$ ppm), 6-fluoroisatin ($\delta = -44$ ppm), 5-fluoroanthranilic acid ($\delta = -60$ ppm), and 6-fluoroanthranilic acid ($\delta = -52$ ppm). Using these values, response measurement for the reaction of 1 mM fluoroisatin to varying concentrations of $ONOO^-$ was conducted (Figure 3). $ONOO^-$ fully converted each

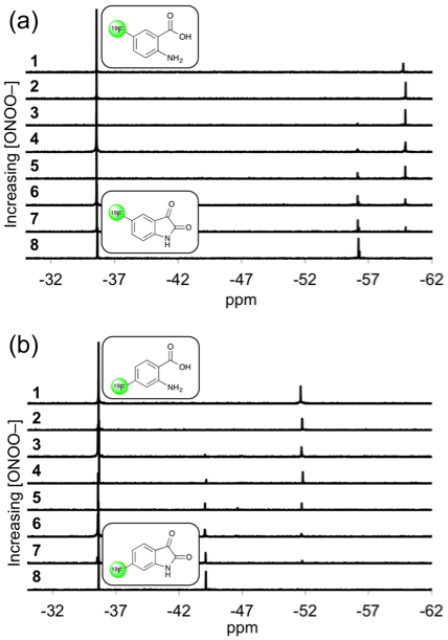


Figure 3: Responses of (a) 5-fluoroisatin and (b) 6-fluoroisatin to $ONOO^-$. 1. 1 mM fluoroanthranilic acid. $(2-7)$ Reaction of 1 mM fluoroisatin with 2. 1.5 mM $ONOO^-$ 3. 1.25 mM $ONOO^-$ 4. 1.0 mM $ONOO^-$ 5. 0.75 mM $ONOO^-$ 6. 0.5 mM $ONOO^-$ 7. 0.25 mM $ONOO^-$ 8. 1 mM fluoroisatin. All reactions were performed in 100 mM HEPES at pH 7.4. Spectra were acquired with 128 scans at 11.7 T using an internal reference peak of trifluoroacetic acid at -35.6 ppm.

fluoroisatin signal to the corresponding anthranilic acid signal at 1.5 equivalent concentration. The resulting chemical shift values of each fluoroisatin to anthranilic acid had the same chemical shift values of the pure samples. The response measurements confirm the hypothesis that fluoroisatins can be used to detect $ONOO^-$ using NMR spectroscopy.

Selectivity Measurement

5-fluoroisatin and 6-fluoroisatin were tested against endogenously present RSON species to evaluate selectivity for $ONOO^-$ detection. For the studies, 500 µM probe was reacted with 1.5 equivalence (750 µM) RSON species, except gluathione, which was reacted at 5 mM (Figure 4). The result

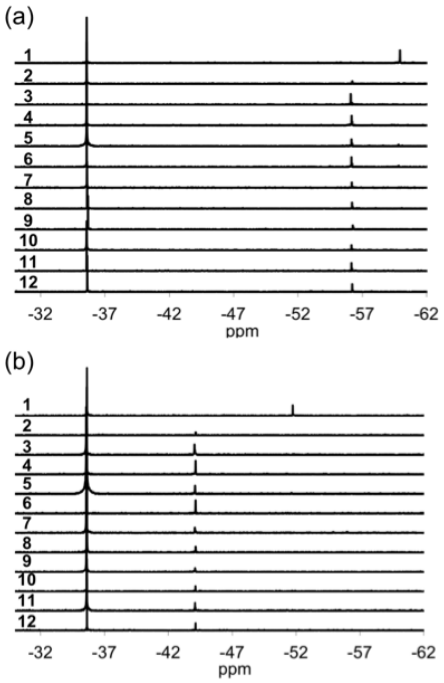


Figure 4: Selectivity of (a) 5-fluoroisatin and (b) 6-fluoroisatin versus 750 µM RSON species, except for GSH, which was reacted at 5 mM. 500 µM fluoroisatin was reacted with 1. $ONOO^-$ 2. ·OH 3. GSH 4. ·NO 5. $Na_2N_2O_3$ 6. KO_2 7. tBuOOH 8. GSNO 9. $NO_2{}^-$ 10. ClO^- 11. $KHSO_5$ 12. H_2O_2. All reactions, except 2, were performed in 100 mM HEPES at pH 7.4. Reaction 2 was performed in 100 mM PBS at pH 7.4 as described previously.[10] Spectra were acquired with 128 scans at 11.7 T using an internal reference peak of trifluoroacetic acid at −35.6 ppm.

show that 5-fluoroisatin and 6-fluoroisatin are highly selective for $ONOO^-$ detection. Only $ONOO^-$ produced an anthranilic acid signal in NMR spectroscopy. In order to show that 5-fluoroisatin and 6-fluoroisatin did react with higher concentrations of RSON species, higher concentration selectivity studies were conducted using H_2O_2 and ·OH (Figure 5 and Figure 6).

The results of these studies confirm that our probe is highly selective for $ONOO^-$. Even at 120 equivalence of H_2O_2, 5-fluoroisatin and 6-fluoroisatin did not fully convert to anthranilic acid. Thus, 5-fluoroisatin and 6-fluoroisatin offer a highly selective detection method for $ONOO^-$.

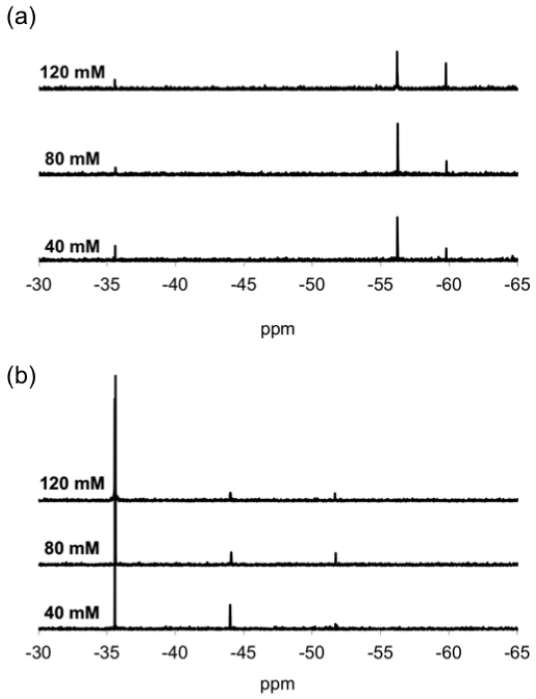


Figure 5: (a) Response of 5-fluoroisatin to high concentrations of H_2O_2. Reaction of 1 mM 5-fluoroisatin with 40, 80, or 120 mM H_2O_2. All reactions were performed in 100 mM HEPES at pH 7.4. Spectra were acquired with 128 scans at 11.7 T using an internal reference peak of 0.001% trifluoroacetic acid at -35.6 ppm. (b) Response of 6-fluoroisatin to high concentrations of H_2O_2. Reaction of 1 mM 6-fluoroisatin with 40, 80, or 120 mM H_2O_2. All reactions were performed in 100 mM HEPES at pH 7.4. Spectra were acquired with 128 scans at 11.7 T using an internal reference peak of 0.1% trifluoroacetic acid at -35.6 ppm.

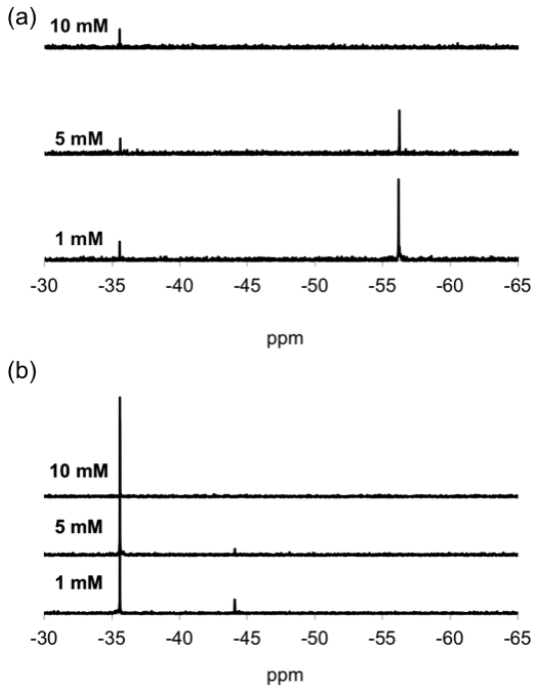


Figure 6: (a) Response of 5-fluoroisatin to high concentrations of ·OH. Reaction of 1 mM 5-fluoroisatin with 1 mM, 5 mM, or 10 mM $Fe(ClO_4)_2$ and H_2O_2. All reactions were performed in 100 mM HEPES at pH 7.4. Spectra were acquired with 128 scans at 11.7 T using an internal reference peak of 0.001% trifluoroacetic acid at −35.6 ppm. (b) Response of 6-fluoroisatin to high concentrations of ·OH. Reaction of 1 mM 6-fluoroisatin with 1 mM, 5 mM, or 10 mM $Fe(ClO_4)_2$ and H_2O_2. All reactions were performed in 100 mM HEPES at pH 7.4. Spectra were acquired with 128 scans at 11.7 T using an internal reference peak of 0.1% trifluoroacetic acid at −35.6 ppm.

HPLC Measurements

HPLC studies were used to confirm the results of the ^{19}F NMR spectroscopy experiments using another instrumentation method. An HPLC method for the separation of isatin and anthranilic acid was found (as described in the Experimental section). Using the established HPLC method, response measurements of 1 mM 5-fluoroisatin to varying concentrations of $ONOO^-$ were conducted (Figure 7). The response measurement and resulting integration of the peaks for 5-fluoroisatin

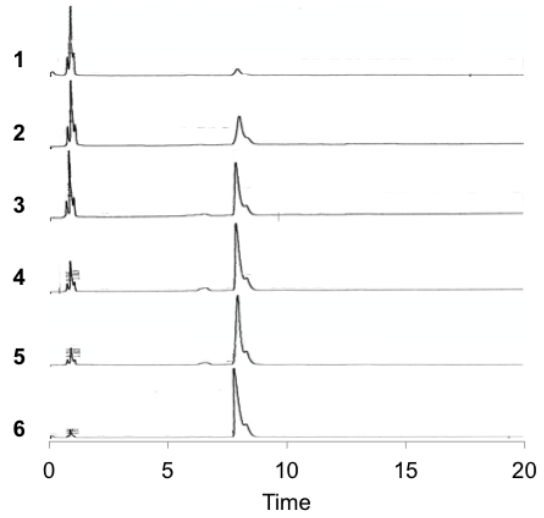


Figure 7: Responses of 5-fluoroisatin to $ONOO^-$. (1-6) Reaction of 1 mM fluoroisatin with 1. 1.5 mM $ONOO^-$ 2. 1.25 mM $ONOO^-$ 3. 1.0 mM $ONOO^-$ 4. 0.75 mM $ONOO^-$ 5. 0.5 mM $ONOO^-$ 6. 0.25 mM $ONOO^-$. All reactions were performed in 20 mM HEPES at pH 7.4.

and 5-fluoroanthranilic acid confirm the results of the ^{19}F NMR spectroscopy experiments. Using integration values, the reaction of isatin with 1.5 equivalence $ONOO^-$ results in an 86% yield of anthranilic acid.

The ^{19}F NMR spectroscopy experiments for the selectivity of 5-fluoroisatin with various RSON species was also confirmed by HPLC (Figure 8). It was necessary to run control HPLC experiments because some RSON species had a trace in the HPLC chromatogram. The selectivity measurements confirm ^{19}F NMR spectroscopy experiments results that 5-fluoroisatin is highly selective for the detection of $ONOO^-$.

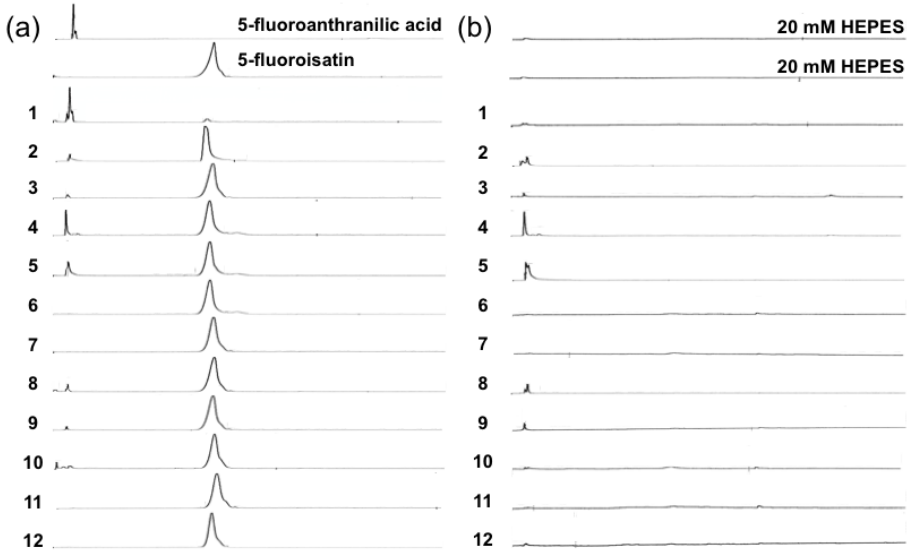


Figure 8: Selectivity of 5-fluoroisatin. (a) HPLC traces of the reaction of 500 µM 5-fluoroisatin with 750 µM various RSON species, except for GSH, which was reacted at 5 mM. 1. $ONOO^-$ 2. ·OH 3. GSH 4. ·NO 5. $Na_2N_2O_3$ 6. KO_2 7. tBuOOH 8. GSNO 9. NO_2^- 10. ClO^- 11. $KHSO_5$ 12. H_2O_2. (b) Blank HPLC traces for 750 µM of various RSON species donors and blanks, except for GSH, which was measured at 5 mM. 1. $ONOO^-$ 2. ·OH 3. GSH 4. ·NO 5. $Na_2N_2O_3$ 6. KO_2 7. tBuOOH 8. GSNO 9. NO_2^- 10. ClO^- 11. $KHSO_5$ 12. H_2O_2.

Cell Testing Results

The response and selectivity measurements indicate that 5-fluoroisatin and 6-fluoroisatin are capable of detecting $ONOO^-$ in cells. Cells were treated with IFN-γ to stimulate $ONOO^-$ production at elevated levels in order to produce a shift in the NMR spectrum. The response and selectivity data indicated that 5-fluoroisatin and 6-fluoroisatin behave similarly in the reaction with $ONOO^-$, so only 5-fluoroisatin was used to demonstrate the detection of $ONOO^-$ in cells. 5-fluoroisatin was incubated in a cellular environment as described in the Experimental section, and then an NMR spectrum was taken for analysis. Replicate experiments were performed to confirm that IFN-γ causes an increase in $ONOO^-$ production and the 5-fluoroisatin is capable of detecting the increase (Figure 9). In order to confirm the increased production of $ONOO^-$ in cells by IFN-γ,

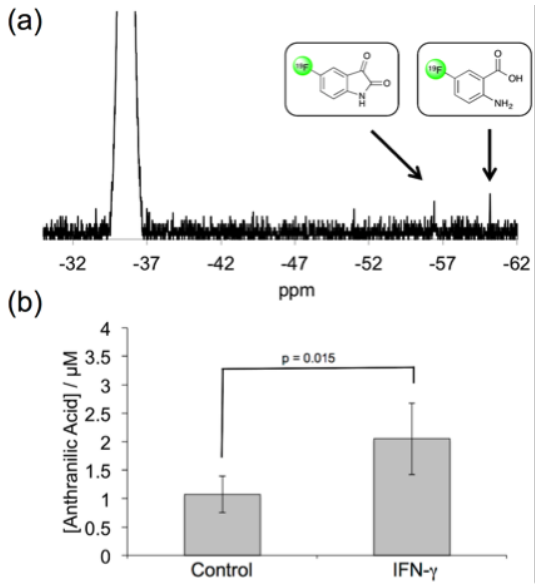


Figure 9: Detection of cellular $ONOO^-$. (a) A549 cells were treated with 50 µM 5-fluoroisatin and 50 ng/mL IFN-γ for 12 hours. The media was transferred to an NMR tube and spectra were acquired with 7300 scans at 11.7 T using an internal reference peak of trifluoroacetic acid at -35.6 ppm. (b) Quantification of the anthranilic acid concentration determined from 19F NMR peak integrations in A549 cells treated for $6-18$ hours with 50 µM 5-fluoroisatin and 0.1% BSA as a vehicle control, n = 5 (Control) or 50 ng/mL IFN-γ, n= 5 (IFN-γ). Statistical analysis was performed using a two-tailed Student's t-test to give p = 0.015. Error bars are ± S.D.

dihydrorhodamine 123 (DHR) was used. DHR was subjected to the same cellular environment as 5-fluoroisatin, and then analyzed for response using fluorescence microscopy (Figure 10). DHR had similar response to IFN-γ stimulated cells, showing an increase in fluorescence in cells exposed

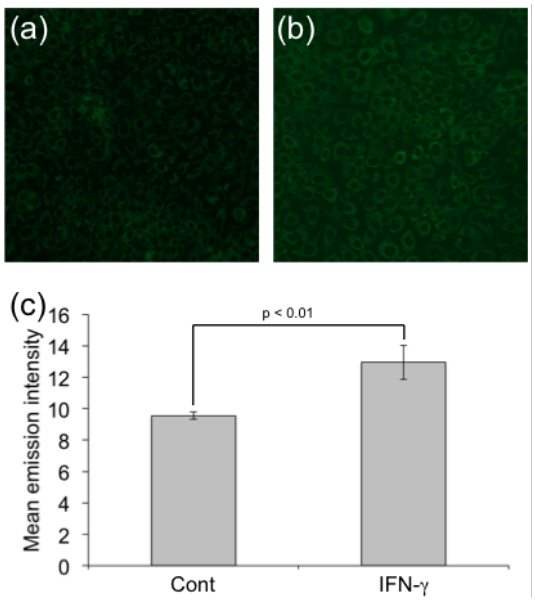


Figure 10: Fluorescence microscopy images of $ONOO^-$ detection in A549 cells using DHR. (a) A549 cells incubated with 10 µM DHR for 30 min at 37 °C, washed and then incubated with a vehicle control for 6 hours. (b) A549 cells incubated with 10 µM dihydrorhodamine 123 for 30 min, washed, and then incubated with 50 ng/mL IFN-γ for 6 hours. (c) Quantification of the mean emission intensity of replicates (n = 3) of experiments described in (a) and (b). Statistical analysis was performed with a two-tailed Student's t-test; error bars are S.D.

to the stimulant. These results confirmed that 5-fluoroisatin was detecting an increased level of $ONOO^-$ in the cellular environment.

Conclusion and Acknowledgements

To conclude, 5-fluoroisatin and 6-fluoroisatin were found to be highly selective and responsive ^{19}F magnetic resonance probes for the detection of $ONOO^-$. A novel oxidative decarbonylation was discovered and utilized for the creation of the probes. 5-fluoroisatin was used to detect $ONOO^-$ in living lung epithelial cells stimulated by IFN-γ.

This research will lead to a better understanding of $ONOO^-$ in biological systems. The creation of two novel ^{19}F magnetic resonance probes will contribute to the growing library of biological detection methods utilizing NMR spectroscopy. The newly discovered oxidative decarbonylation chemistry will lead to a new class of biological probes for the detection of $ONOO^-$.

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